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Comparison of γδ T cell responses and farnesyl diphosphate synthase inhibition in tumor cells pretreated with zoledronic acid

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Exposing human tumor cells to nitrogen-containing bisphosphonates, such as zoledronic acid (Zol), greatly increases their susceptibility to killing by γδ T cells. Based on this finding and other studies, cancer immunotherapy using γδ T cells and nitrogen-containing bisphosphonates has been studied in pilot clinical trials and has shown benefits. Although Zol treatment can render a wide variety of human tumor cells susceptible to γδ T cell killing, there has not been a systematic investigation to determine which types of tumor cells are the most susceptible to γδ T cell-mediated cytotoxicity. In this study, we determined the Zol concentrations required to stimulate half maximal tumor necrosis factor-α production by γδ T cells cultured with various tumor cell lines pretreated with Zol and compared these concentrations with those required for half maximal inhibition of farnesyl diphosphate synthase (FPPS) in the same tumor cell lines. The inhibition of tumor cell growth by Zol was also assessed. We found that FPPS inhibition strongly correlated with γδ T cell activation, confirming that the mechanism underlying γδ T cell activation by Zol is isopentenyl diphosphate (IPP) accumulation due to FPPS blockade. In addition, we showed that γδ T-cell receptor-mediated signaling correlated with γδ T cell tumor necrosis factor-α production and cytotoxicity. Some lymphoma, myeloid leukemia, and mammary carcinoma cell lines were relatively resistant to Zol treatment, suggesting that assessing tumor sensitivity to Zol may help select those patients most likely to benefit from immunotherapy with γδ T cells. (*Cancer Sci* 2013; 104: 536–542)

The majority of human peripheral blood γδ T cells express Vγ2 (also termed Vγ9) and Vδ2 TCR genes[1–4] and show cytotoxicity against a wide spectrum of tumor cells.[5,6] The γδ T cells kill tumor cells through recognition by γδ TCR[7,8] as well as by NK receptors.[9–12] Recent clinical trials have found that Zol, an N-BP, provides clinical benefits when added to standard therapies for patients with mammary carcinoma and multiple myeloma.[13–17] Because N-BPs inhibit FPPS in tumor cells and increase the intracellular level of isopentenyl diphosphate (IPP), leading to the activation of γδ T cells expressing Vγ2Vδ2 TCR,[18–20] it has been suggested that γδ T cells might contribute to the therapeutic effect of Zol in cancer treatment.[21]

Although *in vitro* and *in vivo* studies have shown that Zol renders many types of tumor cells susceptible to γδ TCR-mediated cytotoxicity,[5,15,22–29] there has not been a systematic examination to determine if it would be possible to predict which types of tumors would be most likely to respond to immunotherapy with γδ T cells and Zol. In this study, we have tested a variety of cancer cell lines to determine the Zol concentration required to inhibit FPPS by 50% (as assessed by Rap1A prenylation) and compared these concentrations to those required to stimulate half maximal TNF-α production by γδ T cells cultured with Zol-pretreated tumor cells. We found that the Zol concentrations required for FPPS inhibition closely correlated with those required for stimulation of TNF-α production by γδ T cells but not with the Zol concentrations required to inhibit tumor cell proliferation. Additionally, γδ TCR-mediated signaling correlated with FPPS inhibition.

Materials and Methods

Inhibition of FPPS. Zoledronic acid was purchased from Novartis Pharmaceuticals (Basel, Switzerland) and converted to its sodium salt using an Na^+ form of Dowex 50W×8 (Muromachi Kogyo Kaisha, Tokyo, Japan). Zoledronic acid inhibition of FPPS was determined by assessing the degree of Rap1A prenylation (geranylgeranylation) on Western blotting with varying concentrations of Zol as described in Figure S1.

Derivation of Vγ2Vδ2 T cell lines. Recombinant human IL-2 was kindly provided by Shionogi Pharmaceutical (Osaka, Japan). After institutional review board approval and with written informed consent, PBMC were purified and stimulated with 5 μM Zol and 100 U/mL IL-2 for 10 days as described in Figure S2 to derive Vγ2Vδ2 T cell lines.

Flow cytometry. Flow cytometric analyses were carried out using a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA). The gating strategy is detailed in Figure S2.

Cytokine production. Tumor cells listed in Table S1 were grown, harvested, and resuspended at 1×10^6 cells/0.5 mL in 10-fold serial dilutions of Zol in complete RPMI-1640 media (Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Sigma), 10^{-5} M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), 100 IU/mL penicillin (Meiji Seika Kaisha, Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika Kaisha). After incubation at 37°C with 5% CO_2 for 4 h, the cells were washed three times with 5 mL of the medium and resuspended in 0.5 mL of the same medium. A total of 0.1 mL (2×10^5 cells/well) of the tumor cell suspension was placed on flat-bottomed 96-well plates and 0.1 mL γδ T cells (2×10^5 cells/well) was added (Fig. S2). The plates were incubated at 37°C with 5% CO_2 for 16 h and the culture supernatants stored overnight at −80°C. The samples were then thawed and TNF-α concentrations determined by ELISA (Peprotech, Rocky Hill, NJ, USA) using an ARVO spectrophotometer (PerkinElmer,

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Foster City, CA, USA). All experiments were carried out in triplicate.

Tumor cell growth inhibition assay. Tumor cells listed in Table S1 were grown, harvested, and resuspended at 1×10^4 cells/mL in complete RPMI-1640 medium. A total of 0.05 mL of the cell suspension was added to flat bottomed 96-well plates, followed by 0.05 mL of three-fold serial dilutions of Zol. After incubation at 37°C with 5% CO_2 for 4 h, the supernatant was removed and Zol-free medium added. After an additional 16 h, 0.1 mL CellTiter-Glo reagent (Promega, Madison, WI, USA) was added and the luminescence due to released ATP was measured using an ARVO luminometer (PerkinElmer). All experiments were carried out in triplicate.

γδ T-cell receptor Jurkat transfectant and IL-2 assay. The β^- Jurkat cells expressing Vγ2Vδ2 TCR were prepared and IL-2 release assayed as described previously.[8] Briefly, 2×10^5 TCR transfectant cells in 100 μL were mixed with 2×10^5 tumor cells in 100 μL. Tumor cells were pretreated with serial dilutions of Zol. After 16 h, the supernatants were collected and assayed for IL-2 by their ability to support the proliferation of the IL-2-dependent CTLL-2 cell line. CTLL-2 cell numbers were determined using the CellTiter-Glo reagent as described above. All experiments were carried out in triplicate.

γδ T cell cytotoxicity assay. Tumor cells (1×10^6) were treated with serial dilutions of Zol for 4 h then labeled with 100 μCi $Na^{51}Cr$ for 1 h. The γδ T cells were incubated with the labeled tumor cells (1×10^4 cells/well) at an effector : target ratio of 40:1. Specific ^{51}Cr release was determined as described previously.[5]

Results

High Zol concentrations required for FPPS inhibition in many lymphoma and some myeloid leukemia cell lines *in vitro*. Zoledronic acid inhibits FPPS, rendering tumor cells susceptible to γδ TCR-mediated lysis.[5,30] The Zol inhibition of FPPS results in intracellular accumulation of upstream metabolites such as IPP.[18–20] Downstream metabolites, such as farnesyl diphosphate and geranylgeranyl diphosphate, are depleted leading to the accumulation of unprenylated Rap1A, a small G protein required for cellular adhesion.[31] The accumulation of unprenylated Rap1A was therefore used as a measure of FPPS inhibition. The Zol concentrations required for half maximal inhibition (IC_{50}) of Rap1A prenylation were determined by culturing tumor cell lines with Zol for 16 h and measuring the level of unprenylated Rap1A by Western blotting (Fig. S1). The proportion of tumor cell lines with Zol IC_{50} of 100 μM or greater was 85.7% for lymphoma, 57.1% for myeloid leukemia, and 28.6% for mammary carcinoma cell lines but only 5.8% for the other 52 tumor cell lines (Fig. 1). Of the 52 other tumor cell lines examined, nine had IC_{50} values <10 μM, including the 786-0W and ACHN renal cell carcinoma, the EJ-1 and T24 bladder carcinoma, the MZChA2 bile duct carcinoma, the TGBC1TKB gallbladder carcinoma, the HuO osteosarcoma, the PC-3 prostatic carcinoma, and the HT-1080 fibrosarcoma cell lines.

High Zol concentrations required for γδ T cell activation by Zol-pretreated lymphoma and myeloid leukemia cell lines *in vitro*. We next determined the Zol concentrations required to stimulate half maximal TNF-α secretion (EC_{50}) by γδ T

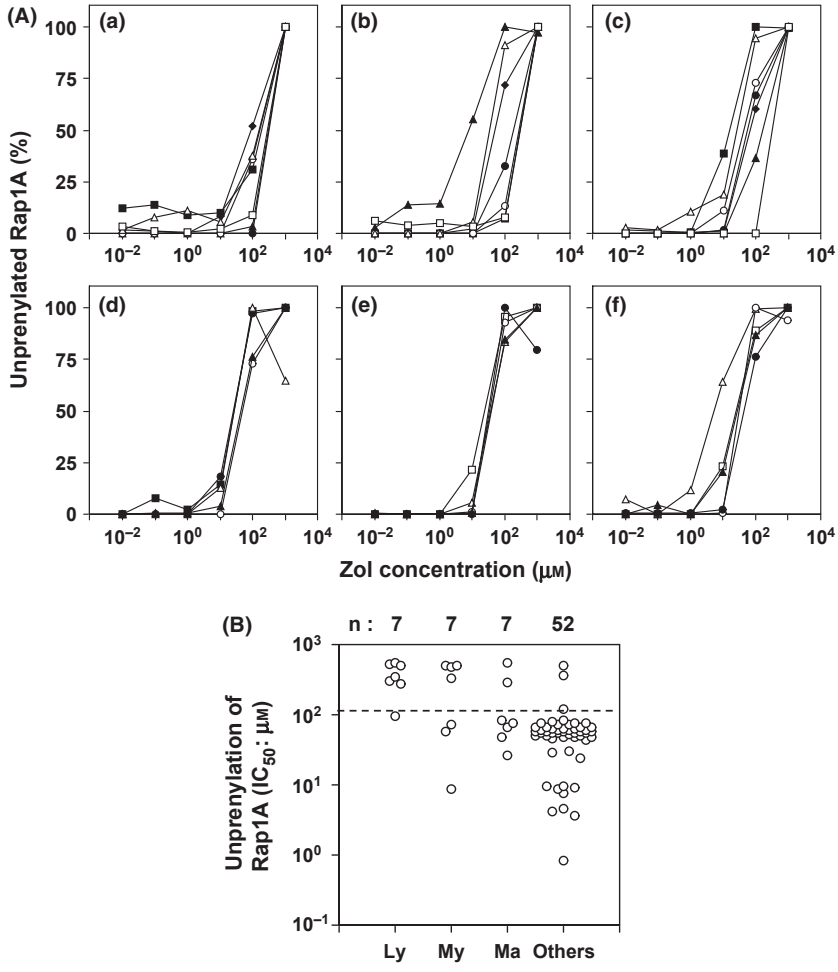

Fig. 1. Differential effects of zoledronic acid (Zol) on farnesyl diphosphate synthase inhibition in tumor cell lines. (A) Dose-dependent Zol inhibition of geranylgeranylation of Rap1A for various types of tumor cell lines: (a) lymphomas, □ MOLT-3, △ PEER, ○ C1R, ♦ J.RT3-T3.5, ■ Raji, ▲ RAMOS-RA1, ● MOLT-4; (b) myeloid leukemias, □ HL60, △ U937, ○ THP-1, ♦ SCC-3, ■ P31/FUJ, ▲ K562, ● NOMO-1; (c) mammary carcinomas, □ YMB-1-E, △ MRK-nu-1, ○ HMC-1-8, ♦ MCF-7, ■ MDA-MB-231, ▲ T-47D, ● SK-BR-3; (d) renal cell carcinomas, ▲ 786-0, △ VMRC-RCZ, ● UOK121, ○ Caki-1, ■ A-704; (e) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ○ KP4-2, □ KP4-3, △ MIAPaCa-2; and (f) other tumor cells, ● TGBC24TKB, ▲ ACS, ○ MG-63, □ LK-2, △ EJ-1. (B) Comparison of Zol concentrations (IC_{50}) required for half maximal inhibition of prenylation of Rap1A in various types of tumor cells. Ly, lymphoma; Ma, mammary carcinoma; My, myeloid leukemia; Others, other tumor cell lines.

cells (Fig. S2) in response to tumor cell lines incubated with Zol (Table S1). The EC_{50} values for most tumor cell lines were between 10 and 100 μM (Fig. 2). The proportion of tumor cell lines with EC_{50} values of 100 μM or greater was 85.7% for lymphoma, 42.9% for myeloid leukemia, and 42.9% for mammary carcinoma cell lines. In contrast, only 3.8% of the other 52 tumor cell lines had EC_{50} values greater than 100 μM. Although both the Daudi Burkitt's lymphoma and the RPMI-8226 plasmacytoma cell lines stimulate γδ T cells through their γδ TCRs, most other lymphoma and myeloid leukemia cell lines stimulated only poor γδ T cell responses *in vitro*, even with exposure to Zol. Some mammary carcinoma cell lines also required high concentrations of Zol to elicit TNF-α responses by γδ T cells. The requirement for relatively high concentrations of Zol for γδ T cell activation shown by some lymphoma, myeloid leukemia, and mammary carcinoma cell lines correlated with the greater Zol concentrations (EC_{50}) required for FPPS inhibition by these cell lines. In contrast, 13 of 73 tumor cell lines had EC_{50} values less than 10 μM, including the ACHN and UOK111 renal cell carcinoma, the EJ-1 bladder carcinoma, the GCIY, KATO III, MKN28, and MKN74 gastric carcinoma, the Saos-2 osteosarcoma, the DLD-1 colorectal carcinoma, the C32TG and G-361 melanoma, the PC-3 prostatic carcinoma, and the HT-1080 fibrosarcoma cell lines.

Inhibition of FPPS closely correlated with TNF-α production by γδ T cells. To assess the degree of correlation between FPPS inhibition and γδ T cell activation, we compared Zol concentrations for FPPS inhibition (IC_{50}) to those for γδ T cell activation (EC_{50}) for each of the tumor cell lines. As shown in Fig. 3, the Zol concentrations required for FPPS inhibition (prenylation inhibition) were well correlated with those required for γδ T cell production of TNF-α. For example, MOLT-3 lymphoma required Zol concentrations of 500 μM for prenylation inhibition and 530 μM for TNF-α production (Fig. 3a). Similarly, BxPC-3 required 55 μM for prenylation inhibition and 58 μM for TNF-α production (Fig. 3g).

Direct cytotoxicity of Zol on tumor cell lines. Because some lymphoma, myeloid leukemia, and mammary carcinoma cell lines were relatively resistant to FPPS inhibition and γδ T cell activation by Zol, we next determined whether direct killing of certain tumor cell lines by Zol was inhibiting their ability to stimulate γδ T cell secretion of TNF-α. As shown in Figure 4(a), a representative sample of tumor cell lines, tumor cell growth inhibition curves were similar. The Zol concentrations required for half maximal tumor cell line growth inhibition (IC_{50}) were similar between the different types of tumors without high variability (Fig. 4b). These findings clearly indicate that the differences in Zol concentrations required for FPPS inhibition and γδ T cell activation were not due to the direct effects of Zol on tumor cell growth. In fact, much higher concentrations of Zol were required to inhibit tumor cell growth than those required to stimulate γδ T cells (Fig. S3). In addition, specific lysis of tumor cells by γδ T cells in the absence of Zol at an effector : target ratio of 1:1 was less than 6%, confirming further that tumor cell viability was not a critical factor determining the difference in IC_{50} and EC_{50} values between different tumor cell types.

γδ T-cell receptor-mediated recognition of Zol-treated tumor cells. We next examined the correlation between γδ TCR-mediated signaling and TNF-α production. Tumor cell lines

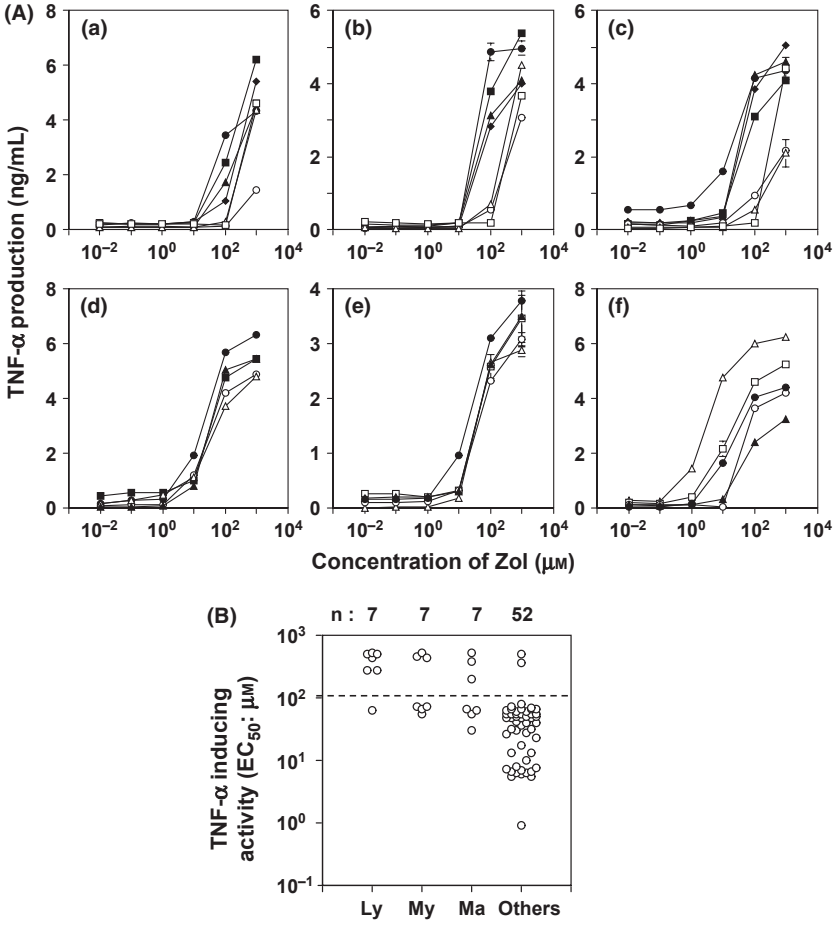

Fig. 2. Comparison of tumor necrosis factor-α (TNF-α) secretion by γδ T cells stimulated with zoledronic acid (Zol)-treated tumor cells. (A) TNF-α production by γδ T cells in response to tumor cells pretreated with various Zol concentrations: (a) lymphomas, □ MOLT-3, △ PEER, ○ C1R, ♦ J.RT3-T3.5, ■ Raji, ▲ RAMOS-RA1, ● MOLT-4; (b) myeloid leukemias, □ HL60, △ U937, ○ THP-1, ♦ SCC-3, ■ P31/FUJ, ▲ K562, ● NOMO-1; (c) mammary carcinomas, □ YMB-1-E, △ MRK-nu-1, ○ HMC-1-8, ♦ MCF-7, ■ MDA-MB-231, ▲ T-47D, ● SK-BR-3; (d) renal cell carcinomas, ▲ 786-0, △ VMRC-RCZ, ● UOK121, ○ Caki-1, ■ A-704; (e) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ○ KP4-2, □ KP4-3, △ MIAPaCa-2; (f) other tumor cells, ● TGBC24TKB, ▲ ACS, ○ MG-63, □ LK-2, △ EJ-1. (B) Comparison of Zol concentrations (EC_{50}) required for half maximal TNF-α secretion by γδ T cells in response to stimulation with different tumor cell lines. Ly, lymphoma; Ma, mammary carcinoma; My, myeloid leukemia; Others, other tumor cell lines.

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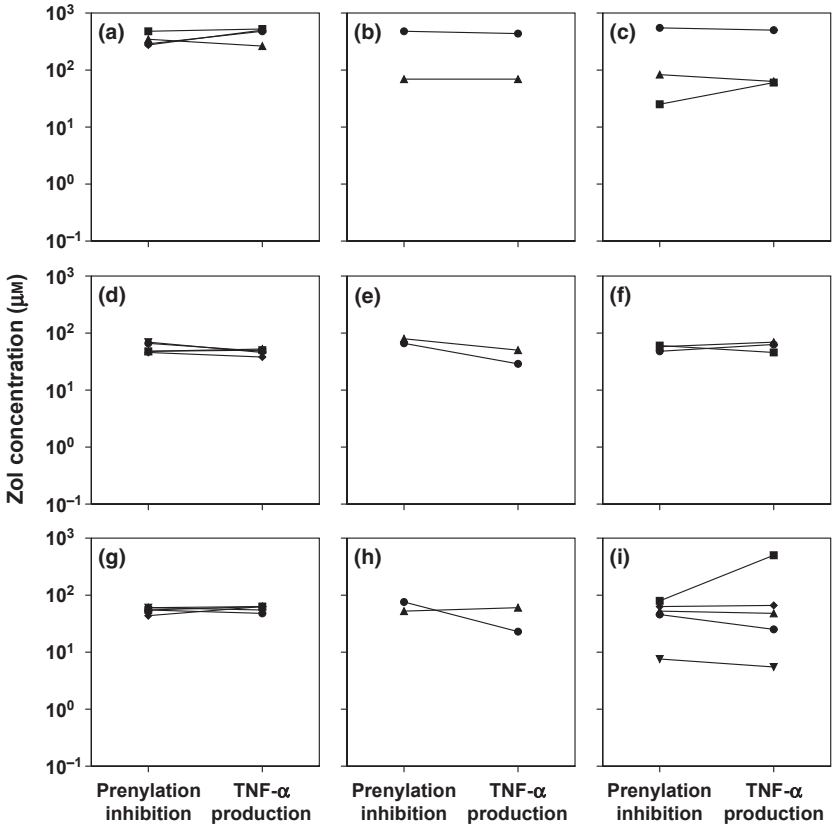

Fig. 3. Correlation between zoledronic acid (Zol) concentrations required for farnesyl diphosphate synthase inhibition and γδ T cell activation. The Zol concentrations required for half maximal inhibition of prenylation of Rap1A and half maximal stimulation of tumor necrosis factor-α (TNF-α) secretion by γδ T cells. Each line connects IC_{50} (prenylation inhibition) and EC_{50} (TNF-α production) for the same tumor cell line: (a) lymphomas, ● C1R, ▲ Raji, ■ MOLT-3, ♦ PEER; (b) myeloid leukemias, ● THP-1, ▲ SCC-3; (c) mammary carcinomas, ● YMB-1-E, ▲ MCF-7, ■ MDA-MB-231; (d) renal cell carcinomas, ● 786-0, ▲ VMRC-RCZ, ■ A-704, ♦ UOK121, ▼ Caki-1; (e) cholangiocell carcinoma, ● TGBC24TKB, ▲ TFK-1; (f) gastric carcinomas, ● ACS, ▲ AGS, ■ MKN1; (g) pancreatic carcinomas, ● BxPC-3, ▲ KP4-1, ■ KP4-2, ♦ KP4-3, ▼ MIAPaCa-2; (h) osteosarcomas, ● HOS, ▲ MG-63; (i) other tumors, ● LK-2, ▲ GCT-IZ, ■ CW-2, ♦ hu2, ▼ EJ-1.

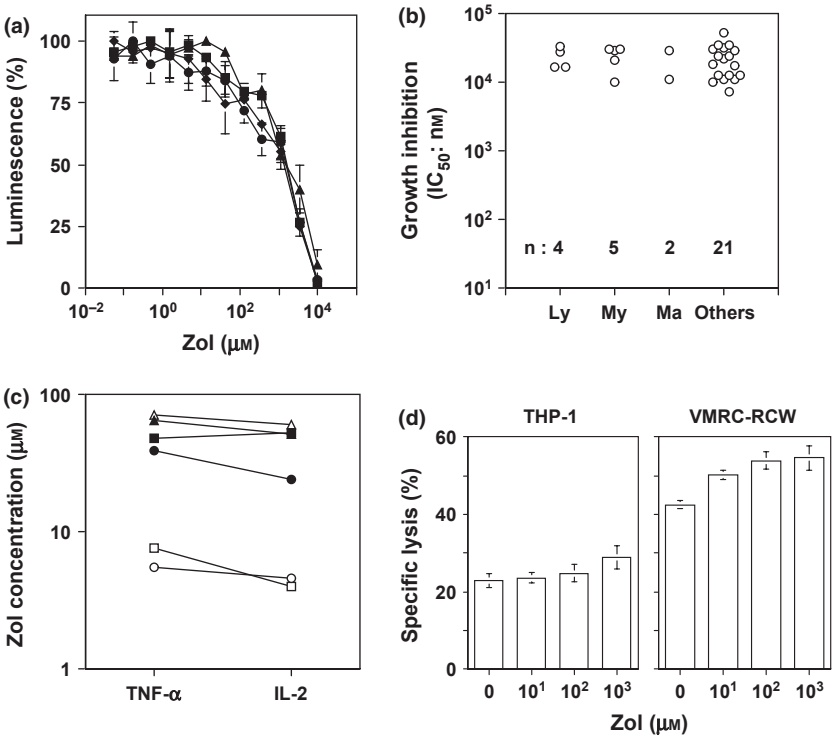

Fig. 4. Correlation between T-cell receptor (TCR)-mediated signaling and cytokine secretion and cytotoxicity by γδ T cells. (a) Dose-dependent inhibition of tumor cell growth by zoledronic acid (Zol). Tumor cell lines were treated with serial dilutions of Zol and cell growth inhibition examined for: ● J.RT3.T3.5; ▲ RAMOS-RAI; ■ Colo320; and ♦ MG63. (b) Comparison of Zol concentrations required for half maximal growth inhibition of various tumor cell lines. Direct inhibition of tumor cell growth by Zol was determined for lymphoma (Ly), mammary carcinoma (Ma), myeloid leukemias (My), and other tumor cell lines (Others). (c) Correlation between tumor necrosis factor-α (TNF-α) production by γδ T cells and γδ TCR-mediated signaling. Zoledronic acid concentrations required for half maximal production of TNF-α by γδ T cells stimulated with Zol-treated tumor cells were compared with those required for half maximal production of interleukin-2 (IL-2) by γδ TCR-expressing J.RT3-T3.5 cells stimulated with Zol-treated tumor cell lines: ● PK-9; ▲ KP4-3; ■ BxPC3; ○ MKN28; △ AGS; and □ G361. (d) Cytotoxic activity of γδ T cells against tumor cells. THP-1 myeloid leukemia and VMRC-RCW renal carcinoma cells were treated with serial dilutions of Zol and used as targets for cytotoxicity by γδ T cells.

were cultured with serial dilutions of Zol and used to stimulate IL-2 production by γδ TCR-expressing Jurkat cells. Because the production of IL-2 requires signaling through the γδ TCR, Zol concentrations required for γδ TCR-mediated signaling can be determined. The Zol concentrations that stimulated half maximal IL-2 production by the transfectants were well correlated with those stimulating half maximal TNF-α secretion by γδ T cells (Fig. 4C). These results show that γδ TCR-mediated signaling is a key factor determining cytokine production by γδ T cells in response to Zol.

γδ T cell cytotoxicity against Zol-treated tumor cells. Because activated γδ T cells express NK receptors such as NKG2D, γδ T cells show cytotoxic activity against tumor cells expressing NK ligands, especially at higher effector to target ratios. Thus, γδ T cells lysed THP-1 myeloid leukemia cells and VMRC-RCW renal carcinoma cells, even in the absence of Zol treatment, at an effector : target ratio of 40:1 (Fig. 4D). This is in stark contrast to cytokine secretion, where γδ T cells did not produce TNF-α in the absence of Zol (Fig. 2). Treating tumor cells with Zol increased γδ T cell killing in a Zol concentration-dependent manner. For THP-1 and VMRC-RCW cell lines, tumor cytotoxicity by γδ T cells was half maximally increased by Zol concentrations of 100–1000 and 5–20 μM, respectively. These values were similar to the Zol concentrations required to stimulate production of TNF-α by γδ T cells (100–1000 μM *vs* 440 μM for THP-1; 5–20 μM *vs* 13 μM for VMRC-RCW). Thus, γδ TCR-mediated recognition of Zol-treated tumor cells is critical not only for cytokine production but also for maximal cytotoxicity.

Discussion

Recent clinical trials have provided evidence that the addition of Zol to the treatment of patients with multiple myeloma and breast cancer provides benefits,[13,14,16,17] although the mechanisms underlying this antitumor activity of Zol have not been determined.[32] One potential mechanism for Zol antitumor activity is the activation of γδ T cells expressing Vγ2Vδ2 TCRs. Zoledronic acid inhibits the FPPS enzyme in isoprenoid synthesis. This results in the accumulation of the upstream metabolite, IPP, that stimulates γδ T cells.[18–20] To date, however, no comprehensive study has been reported comparing the Zol concentrations required for γδ T cell activation to those required for FPPS inhibition in different types of tumors. In this study, we examined 73 human tumor cell lines originating from a variety of tissues to determine the Zol concentrations required for γδ T cell activation and FPPS inhibition. We find that the Zol concentrations required for γδ T cell activation strongly correlated with those for FPPS inhibition. Our findings clearly show that the accumulation of IPP by FPPS inhibition is closely related to the activation of γδ T cells in a variety of different types of tumor cell lines and is consistent with a study restricted to eight breast cancer cell lines.[33] Moreover, signaling through the γδ TCR was required for Zol to stimulate cytokine secretion and maximal cytotoxicity.

Why do different types of tumor cell lines vary in the Zol concentration required for FPPS inhibition and γδ T cell activation? In most tumor cell lines, Zol elicited half maximal γδ T cell responses at 10–100 μM. However, some but not all lymphoma, myeloid leukemia, and mammary carcinoma cell lines required much higher Zol concentrations of 100 μM or more. One possibility is that somatic mutation of FPPS in the cell lines alters their response to inhibition by Zol. There have been 14 mutations in FPPS reported out of 106 cancer samples analyzed (summarized on the Catalogue of Somatic Mutations in Cancer website http://www.sanger.ac.uk/genetics/CGP/cosmic/). However, none of the mutations were found in lymphomas, leukemias, or breast cancers, making this explanation unlikely. As an alternative explanation, we speculate that certain types of tumors require higher concentrations of Zol for FPPS inhibition because Zol is not efficiently taken up through fluid-phase endocytosis[34] due to differences in their metabolism or rate of nutrient uptake. Supporting this hypothesis, the opposite is clearly the case. Lipophilic pyridinium aminobisphosphonates (e.g. BPH-716), which are likely to enter cells more efficiently due to their much higher hydrophobicity, are up to ~12.5-fold more potent activators of γδ T cells than non-lipophilic aminobisphosphonates, such as Zol,[35] despite being 631-fold less potent inhibitors of FPPS.[36]

The addition of Zol to standard treatments for breast cancer patients improved disease-free survival in the subset of patients that have estrogen receptor-positive cancers in a low estrogen environment (either through anti-estrogen treatment or menopause).[13–16] Similarly, improved overall survival was noted with patients with newly diagnosed multiple myeloma.[17] Surprisingly, these improvements were independent of the prevention of skeletal-related events in myeloma[17] and, in the case of breast cancer in postmenopausal women, were related to a decrease in both skeletal and non-skeletal metastases.[15,16] We have shown that the majority of patients with early-stage breast cancer will respond to Zol and many have elevated $V\delta2^+$ T cell frequencies.[37] However, we found in this study that there is heterogeneity in the ability of mammary carcinoma cell lines to stimulate γδ T cell responses, with 42.9% requiring half maximal Zol concentration of >100 μM. If some of the survival benefits of Zol are due to γδ T cells, as has been proposed,[21] this tumor heterogeneity may explain some of the variability of patient response to Zol treatment. Moreover, *in vitro* examination of the ability of a patient's breast cancer cells to stimulate γδ T cells cultured with Zol or the cancer cell's sensitivity to FPPS inhibition by Zol might be useful for selecting patients that would most likely benefit from Zol-based therapy.

As shown in this study, renal cell carcinoma cell lines required relatively low concentrations of Zol to inhibit FPPS and to stimulate γδ T cell responses *in vitro*. Recent clinical trials have shown that γδ T cell/Zol-based therapies provide clinical benefits for patients with lung metastasis of renal cell carcinoma.[38–40] Like the breast cancer and myeloma studies, these observations suggest that the effect of Zol is not solely limited to preventing skeletal metastasis. Instead, Zol may serve to potentiate the effector functions of γδ T cells in patients with a variety of tumor types including those not metastatic to bone. Clinical studies assessing γδ T cell therapy have been primarily carried out in patients with mammary carcinoma, lymphoma, myeloma, renal cell carcinoma, and prostate cancer. However, the correlation between the Zol-sensitivity of a tumor and the clinical outcome of γδ T cell therapy remains unclear. Assessing the *in vitro* sensitivity of tumor cells to Zol may help to predict which tumor types are most likely to respond to therapy and, if cancer cells from individual patients can be tested for Zol sensitivity, aid in deciding which patients to recruit for Zol-based clinical trials. Currently, many laboratories are attempting to develop N-BPs that have affinity for the tumors themselves or that can be targeted to tumors. This medicinal chemistry approach could help to optimize N-BPs for γδ T cell-based cancer immunotherapy.

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Disclosure Statement

C. T. M. is a co-inventor of US Patent 8,012,466 on the development of live bacterial vaccines for activating γδ T cells. The other authors have no conflicts of interest.

Abbreviations

FPPS	farnesyl diphosphate synthase
IL-2	interleukin-2
IPP	isopentenyl diphosphate
N-BP	nitrogen-containing bisphosphonate
NK	natural killer
TCR	T-cell receptor
TNF-α	tumor necrosis factor-α
Zol	zoledronic acid

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Zoledronic acid (Zol) inhibition of geranylgeranylation of Rap1A.

Fig. S2. Flow cytometric analysis of $V\delta2^+$ γδ T cells before and after expansion from PBMC by zoledronic acid/interleukin-2 (Zol/IL-2) and the gating strategy.

Fig. S3. Comparison between zoledronic acid (Zol) concentrations required for γδ T cell responses and tumor cell growth inhibition.

Table S1. List of tumor cell lines used in this study.

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